Copper-dependent autocleavage of glypican-1 heparan sulfate by nitric oxide derived from intrinsic nitrosothiols

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b) running title

S-nitrosylation and heparan sulfate degradation
c) Summary

Cell-surface heparan sulfate proteoglycans facilitate uptake of growth-promoting polyamines [Belting, M., Borsig, L., Fuster, M.M., Brown, J.R., Persson, L., Fransson, L.-Å. and Esko, J.D. (2002) Proc. Natl. Acad. Sci. U.S.A., 99, 371-376]. Increased polyamine uptake correlates with an increased number of positively charged N-unsubstituted glucosamine units in the otherwise polyanionic heparan sulfate chains of glypican-1. During intracellular recycling of glypican-1 there is an NO-dependent deaminative cleavage of heparan sulfate at these glucosamine units, which would eliminate the positive charges [Ding, K., Sandgren, S., Mani, K., Belting, M. and Fransson, L.-Å. (2001) J. Biol. Chem., 276, 46779-46791]. Here, using both biochemical and microscopic techniques, we have identified and isolated S-nitrosylated forms of glypican-1 as well as low-charged glypican-1 glycoforms containing heparan sulfate chains rich in N-unsubstituted glucosamines. The latter were converted to high-charged species upon treatment of cells with 1 mM L-ascorbate, which releases NO from nitrosothiols, resulting in deaminative cleavage of heparan sulfate at the N-unsubstituted glucosamines. S-nitrosylation and subsequent deaminative cleavage were abrogated by inhibition of a Cu\textsuperscript{2+}/Cu\textsuperscript{+}-redox cycle. Under cell-free conditions, purified, S-nitrosylated glypican-1 was able to autocleave its heparan sulfate chains when NO-release was triggered by L-ascorbate. The heparan sulfate fragments generated in cells during this auto-catalytic process contained terminal anhydromannose residues. We conclude that the core protein of glypican-1 can slowly accumulate NO as nitrosothiols while Cu\textsuperscript{2+} is reduced to Cu\textsuperscript{+}. Subsequent release of NO results in efficient deaminative cleavage of the heparan sulfate chains attached to the same core protein while Cu\textsuperscript{+} is oxidized to Cu\textsuperscript{2+}. 
INTRODUCTION

Mammalian glypican-1 (Gpc-1) is a member of a glycosylphosphatidylinositol (GPI)-linked cell-surface proteoglycan (PG) family with six known members to date. Gpc-type PGs, like other cell-surface PG, are selective regulators of ligand-receptor encounters and can thereby control growth and developmental patterning (for reviews, see refs. 1-7). Gpc-type PG contain exclusively the glycosaminoglycan heparan sulfate (HS) attached to sites located close to the C-terminal GPI-membrane anchor. The central part of the protein consists of a cysteine-rich globular domain which contains information that ensures a high level of HS substitution (8). Many of the functions of Gpc are dependent on the polyanionic HS side-chains which are capable of binding and activating a variety of cationic growth factors and cytokines (5, 6).

Cationic polyamines are natural constituents of all cells and essential for growth and differentiation (for review, see ref. 9). Inhibition of endogenous polyamine synthesis, e.g. by inhibition of ornithine decarboxylase (ODC) with α-difluoromethylornithine (DFMO), results in increased polyamine uptake from the environment. We have shown that HS binds the polyamine spermine with an affinity that is 10 times greater than that of DNA (10) and that HS-containing cell-surface PG facilitate uptake of spermine in DFMO-treated cells (11). Mutant Chinese hamster ovary cells deficient in PG synthesis are hypersensitive to growth-inhibition by DFMO and have reduced polyamine uptake (12).

We have previously demonstrated recycling of Gpc-1 in normal fibroblasts and in transformed cells (for review, see ref. 13). During recycling the HS side-chains are degraded and new chains are synthesized on the stubs remaining on the core protein. Biosynthesis of a HS-chain is a complex process that involves many enzymes, including several glycosyl- and
sulfotransferases (for review, see ref. 14). De novo HS assembly is initiated by a unique αGlcNAc-transferase that adds the first GlcNAc to the common glycosaminoglycan-to-protein linkage-region, GlcUA-Gal-Gal-Xyl (15). By the alternating addition of GlcUA and GlcNAc, catalyzed by HS-copolymerases, an extended, linear heparan back-bone is formed. This back-bone is extensively modified by the addition of sulfate groups in various positions and by C-5 uronosyl epimerization. The initial step in HS modification is the regional exchange of an uncharged N-acetyl for a negatively charged N-sulfate on GlcN catalyzed by various isoforms of N-deacetylase/sulfotransferase (NDST). The GlcN residue can also be N-unsubstituted and thereby positively charged (GlcNH$_3^+$). NDST-1, -2 and -4 have an ND/ST-ratio well below 1, whereas NDST-3 has a 10-fold higher deacetylase activity (16). Therefore, expression and participation of this isoform during HS biosynthesis could yield a significant proportion of GlcNH$_3^+$. Alternatively, free amino groups could be generated when biosynthesis is completed, e.g. by N-desulfation catalyzed by sulfamidase or by further N-deacetylation when sulfate donor is no longer available.

We have found that brefeldin A (BFA)-treated cells accumulate a large-size Gpc-1 glycoform with long HS chains containing clusters of GlcNH$_3^+$ residues (17). These clusters are concentrated to the reducing side of heparanase-cleavage sites, especially near the linkage region to the core protein (18). During recycling, the large Gpc-1 glycoform is degraded by peri- and intracellular heparanase, generating HS-oligosaccharides and a glycoform with truncated HS-chains, still containing GlcNH$_3^+$ residues (17, 18). These stubs are further eroded by nitric oxide (NO)-dependent deaminative cleavage at the GlcNH$_3^+$ residues (17). On this glycoform full-size HS-chains can then be resynthesized using the remaining HexUA-terminating stubs as primers. Resynthesis is inhibited by nitrite-deprivation and restored when an NO-donor is supplied (17).
More recently, we have analyzed the effect of intracellular polyamine-depletion on the structure of the HS-Chains in recycling Gpc-1. Cells with upregulated polyamine uptake synthesize HS-Chains with an increased number of both GlcNH$_3^+$ residues and sulfated HexUA, often in close proximity to one another (19). A summary of earlier findings is shown in Scheme 1.

In the present study, we have examined the NO-dependent degradation of HS in more detail. We have focussed on a possible relationship between copper-dependent S-nitrosylation of the Gpc-1 core protein, and subsequent NO-release, and the deaminative cleavage of HS at GlcNH$_3^+$. We have identified and isolated subpopulations containing S-nitroso groups (-SNO) as well as Gpc-1 glycoforms carrying HS with a high content of GlcNH$_3^+$ and studied their processing.
e) Experimental procedures

EXPERIMENTAL PROCEDURES

*Materials*—Cells (human bladder carcinoma cells T24, former code-name ECV304), culture media, sera, antiserum to Gpc-1, brefeldin A (BFA), radioactive precursors, enzymes, prepacked columns, centriplus tubes and other media or chemicals were obtained from sources listed previously (10, 11, 17, 18). α-Difluoromethylornithine (DFMO) was obtained from ILEX Oncology, San Antonio, Texas. Biotin-BMCC, {1-biotinamido-4-[4´-(maleimidomethyl)cyclohexane-carboxamido]butane} was purchased from Pierce, L-ascorbic acid from Sigma and anti-S-nitrosocysteine from Alexis Biochemicals. The polyclonal anti-Gpc-1 serum was the same as that used previously (17). The monoclonal antibody JM-403 specific for HS epitopes containing GlcNH\(^3\) residues (20) was a gift from Dr J. van den Born, University of Amsterdam, NL, and a monoclonal antibody specific for heparin oligosaccharides terminating with anhydromannose (anMan) or anhydromannitol (anManOH) was a gift from Dr G. Pejler, Swedish Agricultural University, Uppsala, SE (21). As secondary antibodies we used either FITC-labeled or Texas Red-labeled goat antirabbit IgG (Molecular Probes) or FITC-labeled rabbit antimouse Ig from Sigma.

*Cell Treatments and Radiolabeling*—Cells were maintained as described (10, 11, 18) and preincubated with the appropriate medium before radiolabeling. Pretreatments with 5 mM DFMO and 1 μM spermine were carried out as described (11). Radiolabeling was carried out using 20 μCi/ml D-[6-\(^3\)H]glucosamine and/or 50 μCi/ml of \([^{35}\text{S}]\)sulfate in sulfate-poor medium (17, 18). Cells were generally labeled for 24 h either while attaining confluence or thereafter.
Extraction, Isolation and Separation of PG Glycoforms and Core Proteins- Cells were extracted with radioimmune precipitation buffer (RIPA) followed by immunoisolation of all Gpc-1 glycoforms using anti-glypican-1 antiserum as described previously (17). Cells were also extracted with Triton X-100 and PG were recovered by passage over DEAE-cellulose or by desalting on PD-10 (17, 18). Separation of PG glycoforms and various degradation products was performed by gel-permeation chromatography on Superose 6, or by ion exchange chromatography on MonoQ (17, 18).

Degradation and Modification Procedures- HS chains and chain stubs were released from the core protein by treatment with alkaline borohydride (17, 18). Enzymatic degradations of HS were performed with HS lyase and deaminative cleavage at GlcNH₃⁺ with HNO₂ at pH 3.9 as previously described (17, 18). Radioactivity measurements, buffer changes, concentrations and recovery procedures as well as carriers were the same as decribed previously (17, 18).

S-nitrosylated Glypican- S-nitrosylated glypican was detected by adapting the method of Jaffrey et al. (22). Cells were metabolically labeled with D-[6-³H]glucosamine and [³⁵S]sulfate in the absence or presence of 0.5 mM sodium nitroprusside and 0.01 mM CuCl₂. After extraction of the cells with 2.5% SDS in neocuproine-containing buffer, blocking of free thiols with 2 mM N-ethylmaleimide and desalting, samples were biotinylated with biotin-BMCC in the absence or presence of 1 mM L-ascorbate according to the description of the manufacturers. Biotinylated material was recovered on streptavidine-agarose and eluted with 4 M guanidine-HCl. After dialysis against RIPA buffer, Gpc-1 was immunoisolated (17). S-nitrosylated glycoforms were also isolated using anti-SNO-cysteine antiserum using the same method as for Gpc-1 (17).

Confocal laser scanning immunofluorescence microscopy- Cells were seeded at a concentration of 20,000 cells/well in chamber-slides with covers (LabTek) and cultured in
MEM overnight to obtain sparse cultures. Cells were left untreated or treated with 1 mM L-ascorbate for 1 h and then washed with PBS three times, fixed and permeabilized by treatments with acetone for 2-4 min followed by incubation with 1 ml 2% (v/v) H$_2$O$_2$ in 60% (v/v) methanol for 15 min. After washing with water 3 x 1 min, cells were incubated with non-immune serum (1:100 dilution) for 30 min at room temperature. Primary antibodies were applied, anti-SNO as described by the manufacturer, JM-403 at 1:1000 dilution and anMan-specific antibody at 1:100 dilution and kept for 3 h. Cells were then washed three times with PBS and exposed to secondary antibodies (1:500 dilution) for 2 h. Before microscopy the slides were washed again with PBS and air-dried. Controls with pre-immune serum or with omission of either primary or secondary antibody were always run.

Fluorescence images were obtained by using confocal laser scanning equipment (MRC-1024; Bio-Rad) attached to a Nikon Eclipse E800 upright microscope using the oil-immersion objective. Excitation was obtained with an Ar laser at 488 nm for FITC and at 560 nm for Texas Red and the emitted light was filtered with an appropriate long-pass filter (cutoff, 540 nm and 605 nm, respectively). The images shown were obtained at a focal plane that was at the center of the cell and of 0.3-0.5 μm thickness. Images were digitized and transferred to Adobe PhotoShop for merging, annotation and printing.
RESULTS

Glypican-1 Glycoforms Containing Heparan Sulfate with High or Low Content of \textit{N}-unsubstituted Glucosamine- We have previously demonstrated that Gpc-1 can contain HS chains with clusters of GlcNH$_3^+$ residues, especially near the linkage region to the core protein (18); see also Scheme 1. In Gpc-1 synthesized by polyamine-depleted cells, GlcNH$_3^+$ residues were found also in more distal regions of the HS chains (19). An increased number of GlcNH$_3^+$ units reduces the net negative charge of a HS chain and provides an increased number of cleavage sites for endogenously generated NO/nitrite. NO-Dependent deaminative cleavage converts the positively charged GlcNH$_3^+$ to a neutral anhydromannose (anMan) residue, located at the reducing terminus of the released HS-fragment. Thus, the remaining protein-bound HS chains become shorter and will have a greater net negative charge density than the undegraded chains.

To search for Gpc-1 glycoforms with different net charge densities, we immunoisolated metabolically radiolabeled Gpc-1 produced by subconfluent untreated cells and by subconfluent cells exposed to DFMO to upregulate spermine uptake and GlcNH$_3^+$ formation (19). NO, which induces the deaminative degradation of HS at GlcNH$_3^+$, can be stored in proteins bound to cysteines as SNO groups (23). As NO can be released from SNO groups by treatment with L-ascorbate (22), we also isolated Gpc-1 produced by subconfluent cells that were exposed to L-ascorbate during up-regulation of GlcNH$_3^+$ formation by DFMO.

Total Gpc-1 from untreated cells afforded two major radiosulfated PG pools of either low negative charge density (I), or high negative charge density (III) and one minor pool of intermediate charge density material (II) upon ion exchange FLPC on MonoQ (Fig. 1 A).
Total Gpc-1 from cells treated with DFMO yielded larger amounts of the low and intermediate negatively-charged glycoforms (pools I and II in Fig. 1 B) than of the high negatively-charged form (pool III in Fig. 1 B). In contrast, when ascorbate was included, the low and intermediate negatively-charged forms (pool I-II) were much reduced and the high negatively-charged glycoform (pool III) was the major one (Fig. 1 C).

To examine the size and the GlcNH$_3^+$ content of the HS-chains in the Gpc-1 charge variants (Fig. 1 A-C), chains were released by alkali treatment and chromatographed on Superose 6 before and after deaminative cleavage (Fig. 2). HS-chains from pool I in Fig. 1 A comprised both large and small chains (Fig. 2 A), whereas chains from pool III in Fig. 1 C were only small (Fig. 2 C). Corresponding results were obtained with chains from the other pools I and III in Fig. 1 A-C; chains from pools II gave intermediate patterns (data not shown). To estimate the content of GlcNH$_3^+$, the alkali-released HS-chains were treated with HNO$_2$ at pH 3.9 and rechromatographed. The long HS-chains from a low negatively-charged Gpc-1 (pool I in Fig. 1 A) were extensively degraded (Fig. 2 B; cf. Fig. 2 A), whereas chains from a high negatively-charged glycoform (pool III in Fig. 1 C) were only marginally degraded (Fig. 2 D; cf. Fig. 2 C). Corresponding results were obtained with chains from the other pools I and III in Fig. 1 A-C (data not shown). These results confirm that low-negatively charged Gpc-1 glycoforms carry HS chains with a large number of GlcNH$_3^+$ residues.

We also examined the Gpc-1 precursor to the recycling, partially degraded forms. The precursor was obtained after treatment with BFA (17-19), either from confluent cells with ongoing polyamine synthesis or from cells with inhibited endogenous synthesis, and chromatographed on MonoQ (Fig. 3 A and B). Both of them yielded broad elution profiles with no distinct separation into differently charged glycoforms. This is because their long side-chains have not been subject to degradation by heparanase or by NO/nitrite (17-19). However, as expected, the BFA-PG from DFMO-treated cells, which has a higher content of
GlcNH\textsubscript{3}\textsuperscript{+} than the PG from cells with undisturbed polyamine synthesis, yielded an elution profile that was displaced toward a lower ionic strength (Fig. 3 B).

Formation of Gpc-1 glycoforms containing HS chains with very high GlcNH\textsubscript{3}\textsuperscript{+} content was thus promoted by inhibition of endogenous polyamine synthesis (Fig. 1 B and 3 B). Furthermore, subsequent conversion to glycoforms with a low content of GlcNH\textsubscript{3}\textsuperscript{+} (Fig. 1 C) appeared to be generated by NO released from SNO groups by ascorbate. The SNO groups could either be situated in the Gpc-1 core protein or in other proteins. However, the central domain of Gpc core proteins contains 14 conserved cysteines, some of which could be potential sites for S-nitrosylation (1, 8).

Deaminative Cleavage of Glypican-1 Heparan Sulfate by Nitric Oxide Derived from Nitrosothiols- To test whether de novo synthesis of NO from arginine and generation of free nitrite were required for cleavage at the GlcNH\textsubscript{3}\textsuperscript{+} residues in HS of Gpc-1, the NOS-inhibitor nitro-arginine and the nitrite-quencher sulfamate were added to cells. As shown in Fig. 1 D, ascorbate-generated conversion of the low-charged glycoforms into the high-charged ones was abrogated neither by the NOS-inhibitor nor by sulfamate indicating that de novo formation of NO was not required and suggesting that NO acted directly on HS without a nitrite intermediate.

The content of SNO groups in proteins is dependent on the Cu\textsuperscript{+}/Cu\textsuperscript{2+} ratio, which controls the relative formation and decomposition kinetics (24, 25). As the Cu ions may participate in a redox cycle, we used neocuproine to selectively chelate Cu\textsuperscript{+} and inhibit redox cycling. Cells treated in this manner synthesized mainly a low-charged glycoform (pool I in Fig. 1 E), whereas the intermediate and high-charged glycoforms were much reduced (pools II-III in Fig. 1 E) indicating that NO-dependent deaminative cleavage of HS was prohibited by chelation of Cu\textsuperscript{+}. 


To test whether conversion of the low-charged to the high-charged glycoform could be stimulated by supplementation with Cu ion, cells were treated with Cu$^{2+}$. The result showed that conversion of low-charged to high-charged forms was increased (Fig. 1 F) confirming that an endogenous Cu$^{+}$/Cu$^{2+}$ redox cycle supported S-nitrosylation and NO-release, resulting in subsequent deaminative cleavage of HS. However, ascorbate-generated release of NO appeared to be independent of Cu ions, as conversion of low-charged to high-charged Gpc-1 was not inhibited by neocuproine (Fig. 1 G). Ascorbate was probably oxidized to dehydroascorbate.

Copper-Dependent S-Nitrosylation of Glypican-1 and Autocatalyzed Deaminative Cleavage of Heparan Sulfate- To search for the presence of intrinsic nitrosothiols in the Gpc-1 core protein, we subjected $[^{35}\text{S}]$sulfate-labeled material from untreated cells to thiol-specific biotinylation in the absence or presence of ascorbate. Biotinylated material was isolated and checked for the presence of immunoreactive, radiolabeled Gpc-1. Significant yields of such material could only be recovered when ascorbate was included during the biotinylation procedure. Conversely, we also used anti-S-nitrosocysteine to isolate S-nitrosylated Gpc-1 from the total immunoisolated Gpc-1 pool. In the case of Gpc-1 produced by subconfluent untreated cells (Fig. 1 A), SNO-containing Gpc-1 amounted to approx. one-third of the total population. In the case of DFMO-treated cells (Fig. 1 B), SNO-positive material amounted to approx. one-fifth. When SNO-containing Gpc-1 from polyamine-synthesis inhibited cells was subjected to ion exchange FPLC on MonoQ, it eluted in pool III (Fig. 1 H), indicating that it contained HS chains that had already been processed by deaminative cleavage at the GlcNH$_3^+$ residues. Thus, the results obtained so far suggested that formation of free amino groups preceded S-nitrosylation and that S-nitrosylation of newly-made Gpc-1 was slow, whereas NO-release and subsequent deaminative cleavage were rapid.
The BFA-arrested Gpc-1 precursor with long GlcNH$_3^+$-containing HS-chains (18) is poorly S-nitrosylated$^2$. Furthermore, in polyamine-deprived cells, the GlcNH$_3^+$-content of the HS chains in this Gpc-1 glycoform can be further increased (Fig. 3 B and ref. 19). To examine whether purified, BFA-arrested Gpc-1 PG isolated from DFMO-treated cells could support autocleavage of its HS-chains, we treated this PG with NO-donor and Cu$^{2+}$ in the absence or presence of ascorbate under cell-free conditions. The PG, which was purified on MonoQ and eluted in the void volume of Superose 6 (results not shown), remained undegraded after S-nitrosylation by using NO-donor and Cu$^{2+}$ (Fig. 4 A). When NO was subsequently released from the SNO groups by ascorbate, it was degraded into HS-oligosaccharides and a PG with truncated HS-chains (Fig. 4 B). Further treatment with alkali to release the HS stubs revealed the full extent of HS-chain depolymerization (Fig. 4 C). When the covalent connection between the HS-chains and the core protein of Gpc-1 were severed by alkali treatment prior to S-nitrosylation, HS was insensitive to degradation, even by repeated additions of NO-donor, Cu$^{2+}$ and ascorbate (Fig. 4 D). As a control, the released HS chains were treated with HNO$_2$ at pH 3.9 (Fig. 4 E). It is seen that the extent of depolymerization caused by autocleavage of HS attached to the core protein (Fig. 4 C) and that obtained by the conventional deaminative cleavage (Fig. 4 E) was similar. These results thus supported the notion that NO stored as nitrosothiols in the Gpc-1 core protein was released by ascorbate and was catalyzing the cleavage of the HS-chains. If the agent (NO) and the substrate (HS) were part of the same molecule (Gpc-1), the reaction should be concentration-independent. It is unlikely that NO was derived from a contaminating NO-donor, as this component would have to be strongly bound to Gpc-1 and resist dissociation by both urea and guanidine during purification of Gpc-1 by ion exchange and gel chromatography. It is remarkable that the S-nitrosylation/-denitrosylation capacity could be restored after this treatment.
Sequential Cu$^{2+}$-loading, S-nitrosylation, NO-release and Autocatalyzed Deaminative Cleavage of Heparan Sulfate- We can conclude that Cu$^{2+}$ ions, NO-donor and ascorbate are all needed to sustain efficient depolymerization of HS in Gpc-1 (Fig. 4). When copper ions were chelated (Fig. 1 E and F) or when ascorbate (Fig. 4 A) or NO-donor (cf. Fig. 5 A and B) were omitted, the reaction was abolished or greatly impeded. The partial reaction in Fig. 5 B could be due to the presence of some SNO-groups in the starting material or to the generation of free radicals by copper ion and ascorbate. Preloading of purified Gpc-1 with Cu$^{2+}$ prior to S-nitrosylation/denitrosylation, and denitrosylation of preformed S-nitrosylated Gpc-1 resulted in a similar extensive depolymerization of HS (Fig. 5 C and D). Ascorbate treatment of S-nitrosylated Gpc-1 recovered by using anti-SNO antiserum resulted in an incomplete degradation (Fig. 5 E), probably because the reaction was initiated when immunoisolated Gpc-1 was bound to the Protein A-Sepharose beads. These results thus suggest that there may be Cu$^{2+}$-binding sites in the Gpc-1 core protein and they lend further support to the notion that NO released from intrinsic nitrosothiols can catalyze deaminative cleavage of HS chains in the same Gpc-1 molecule.

Immunofluorescence Microscopy Visualization of NO-release from Nitrosothiols, Deaminative Cleavage of Heparan Sulfate and Formation of Degradation Products- To visualize the autocatalytic processing of HS in cells, we used confocal scanning immunofluorescence microscopy. SNO groups detected by a polyclonal antiserum (Fig. 6 A, SNO-Cys) and HS chains containing GlcNH$_3^+$ residues (Fig. 6 B, JM-403) colocalized distinctly, especially in compartments closely encircling the nucleus (Fig. 6 C, Merged), indicating that a substantial portion of resident, intracellular Gpc-1 is both S-nitrosylated and carries HS chains with GlcNH$_3^+$ residues. Total Gpc-1 had the same intracellular distribution (Fig. 5 D, GPC, red).
The SNO-containing epitope was almost completely obliterated upon ascorbate treatment (Fig. 6 A, SNO-Cys + Ascorbate). When NO-donor and Cu\(^{2+}\) were provided after ascorbate treatment, the SNO-containing epitope was partially restored (Fig. 6 A, SNO-Cys + Ascorbate + SNP + CuCl\(_2\)). The GlcNH\(_3^+\)-containing epitope, which was prominent in untreated, subconfluent cells (Fig. 6 B, JM-403), disappeared upon exposure to ascorbate (Fig. 6 B, JM-403 + Ascorbate). Concomitantly, HS-oligosaccharides terminating with anMan appeared (Fig. 6 B, AM + Ascorbate). The latter were localized to vesicles closely encircling the nuclei. No anMan-containing HS-oligosaccharides were detected in untreated, subconfluent cultures (Fig. 6 D, AM, no green). These results demonstrated intracellular cleavage of HS at GlcNH\(_3^+\), generated by NO that was released from SNO groups by ascorbate, and concomitant production of HS-oligosaccharides terminating with anMan.
DISCUSSION

The present results show that Gpc-1 is another member of the constantly expanding class of proteins that can be functionally modified by S-nitrosylation (26). The Gpc-1 core protein contains, in addition to 14 conserved Cys, several Asp and His residues in the central domain (27), which could form the appropriate acid-base motifs in the tertiary structure (26). The Gpc-1 core protein also contains several separate His residues that could form Cu\(^{2+}\)-binding sites. The presence of S-nitrosylated Gpc-1 was also demonstrated by confocal laser-scanning immunofluorescence microscopy which showed strong colocalization between S-nitrosothiols, Gpc-1 and HS epitopes comprising GlcNH\(^3\)+ residues.

We conclude that NO-release from intrinsic nitrosothiols in the Gpc-1 protein initiates deaminative cleavage at the GlcNH\(^3\)+ residues of the HS-chains, generating chain-fragments or oligosaccharides terminating with anMan. As blocking of Cu\(^+\)/Cu\(^{2+}\) redox cycles inhibit S-nitrosylation and indirectly abrogates deaminative cleavage of HS, we propose that Gpc-1 undergoes S-nitrosylation/-denitrosylation as shown in Scheme 2. Cys residues in the globular part of the Gpc-1 core protein become S-nitrosylated with NO in a Cu\(^{2+}\)-dependent reaction. It is possible that SNO groups form a non-covalent dipol-ion interaction with GlcNH\(^3\)+ residues in the HS-chains. It is noteworthy that Gpc-1 glycoforms with long GlcNH\(^3\)+-rich HS-chains cannot be quantitatively immunoprecipitated with anti-Gpc antiserum (18). Recovery by immunoprecipitation is improved several-fold if the HS-chains are removed, e.g. by treatment with HS lyase, or if NO is released by treatment with ascorbate.\(^3\)

Although NO was released from SNO groups by ascorbate in these studies, the natural triggering mechanism remains unknown. Released NO may act directly on the GlcNH\(^3\)+
residues, particularly if nitroxy anion (NO-) is the major species generated, which could be supported by oxidation of Cu⁺ to Cu²⁺ completing the redox cycle (Scheme 2). The Gpc-1 core protein can thus store NO in a way that is reminiscent of that of hemoglobin, which can also take up and store NO as SNO. NO is later released when hemoglobin is deoxygenated (28).

The HS chains of Gpc-1 are subject to extensive degradation and remodelling during intracellular recycling (11, 19). When cells are treated with the ODC-inhibitor DFMO, endogenous polyamine synthesis diminishes and uptake from the environment is increased. This is correlated with an increase in the number of GlcNH₃⁺ residues, i.e. NO-sensitive cleavage sites (see Scheme 1). Polyamine-deficiency may somehow affect HS metabolism by signaling to the biosynthetic machinery (upregulation of NDST-3 and 2-OST?) or to the degradative pathway (induction/activation of sulfamidase?). In either case, there will be more GlcN residues with free, positively charged amino group. The results obtained in the present study show that, under such conditions, HS-chains in Gpc-1 can become so extensively modified that the affinity for the anion exchanger MonoQ is markedly diminished, permitting the isolation of separate and distinct charge glycoforms. Presumably, all three HS-chains need to display a substantial, simultaneous increase in their GlcNH₃⁺-content to generate charge glycoforms that can be separated from other Gpc-1 glycoforms.

Self-processing of glypican, induced by NO-release from intrinsic SNO groups, may be a critical step in the uptake and targeting of many different types of polyelectrolytes, including polyamines (11, 12, 29). Hence, knowledge obtained by studying polyamine uptake may be useful as a model for the design of synthetic DNA delivery systems (30). Glypicans with HS side-chains that can be induced to increase their poly-zwitterionic character could be versatile carriers.
REFERENCES


(i) Footnotes

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1 The abbreviations used are: anMan, anhydromannose; BMCC, 1-biotinamido-4-[4´-(maleimidomethyl)cyclohexane-carboxamido]butane; BFA, brefeldin A; DFMO, α-difluoromethylornithine; GlcNAc, N-acetylglucosamine; GlcNH₃⁺, N-unsubstituted glucosamine; GlcN, glucosamine with unspecified N-substituent; GlcNSO₃, N-sulfamidoglucosamine; GlcUA, D-glucuronic acid; Gpc, glypican; GPI, glycosyl-phosphatidyl-inositol; HexUA, unspecified hexuronic acid; HS, heparan sulfate; NDST, N-deacetylase/sulfotransferase; ODC, ornithine decarboxylase; -OSO₃, O-sulfate; OST, O-sulfotransferase; PG, proteoglycan; RIPA, radioimmune precipitation buffer; SNO, S-nitroso group; SNP, sodium nitroprusside.

2 Cheng, F., Mani, K., van den Born, J., Ding, K., Belting, M. and Fransson, L.-Å., unpublished observations

3 Cheng, F. and Ding, K., unpublished observations

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FIG. 1. **Separation of low negatively-charged and high negatively-charged glypican-1 glycoforms by ion exchange FPLC on MonoQ.** Cells (T-75 cm² dishes) were incubated with [³⁵S]sulfate for 24 h while attaining confluence in the absence (A) or presence of 5 mM DFMO and 1 µM spermine (B), 5 mM DFMO, 1 µM spermine and 1 mM L-ascorbate (C), 5 mM DFMO, 1 µM spermine, 1 mM L-ascorbate and 10 mM nitro-arginine and 10 mM sulfamate (D), 0.01 mM neocuproine (E), 0.01 mM CuCl₂ (F) and 5 mM DFMO, 1 µM spermine, 1 mM L-ascorbate and 0.01 mM neocuproine (G). Radiolabeled total Gpc-1 was immunoisolated from detergent extracts (RIPA) of the cells and then chromatographed on MonoQ. In (H) cells were incubated as in (B), total Gpc-1 was immunoisolated, reconstituted into RIPA and then the SNO-containing subpopulation was immunoisolated using anti-nitrosocysteine and chromatographed on MonoQ. Elution was performed with a linear gradient from 0.3 M NaCl (tube 15) to 1.2 M NaCl (tube 70). ³⁵S (■—■).

FIG. 2. **Identification of glucosamines with free amino group in HS from low negatively-charged (A, B) and high negatively-charged (C, D) glypican-1 glycoforms.** HS-chains were released from the low-charged Gpc-1 glycoform of untreated cells (pool I in Fig. 1 A) and from the high-charged glycoform of cells treated with DFMO, spermine and ascorbate (pool III in Fig. 1 C) by using alkaline borohydride. The chains were then chromatographed on Superose 6 before (A and C, respectively) and after deaminative cleavage at pH 3.9 (B and D, respectively). ³⁵S (■—■); V₀, void volume; Vₜ, total volume.
FIG. 3. Ion exchange FPLC on MonoQ of BFA-arrested PG from untreated T24 cells (A), and T24 cells exposed to DFMO and spermine (B). Confluent cells (T-75 cm² dishes) were incubated with [³H]glucosamine and [³⁵S]sulfate for 24 h in the presence of 10 µg/ml BFA. Cells were extracted with Triton X-100 and the total PG pool was recovered by passage over DEAE-cellulose. After gel permeation chromatography on Superose 6, where the PG eluted in and near the void volume (data not shown), the PG were subjected to ion exchange FPLC. Elution was performed with a linear gradient from 0.3 M NaCl (tube 15) to 1.2 M NaCl (tube 70). ³H (o-----o); ³⁵S (■——■).

FIG. 4. Auto-catalyzed deaminative cleavage of [³H]HS in purified BFA-arrested PG from DFMO-treated T24 cells. Polyamine-deprived cells were treated with BFA as described (19) and [³H]glucosamine-labeled BFA-PG was isolated and purified on MonoQ (Fig. 3 B). It chromatographed in the void volume of Superose 6 (data not shown). Samples of this large-size PG were then rechromatographed after a 1 h-incubation at room temperature with 0.5 mM sodium nitroprusside (SNP, NO-donor) and 0.01 mM CuCl₂ (A), after a 1 h-incubation with 0.5 mM SNP, 0.01 mM CuCl₂ and 1 mM L-ascorbate (B), after the same treatment followed by release of the HS-chains from the core protein by alkaline elimination (C), after release of the HS-chains from the core protein by alkaline elimination followed either by repeated (4 times) incubations with 0.5 mM SNP, 0.01 mM CuCl₂ and 1 mM L-ascorbate (D), or treatment with HNO₂ at pH 3.9 (E). ³H (o-----o).
FIG. 5. Auto-catalyzed deaminative cleavage of $[^3$H]HS in purified BFA-arrested PG after sequential Cu$^{2+}$-loading, S-nitrosylation and ascorbate-generated NO-release. Polyamine-deprived cells were treated with BFA and $[^3$H]glucosamine-labeled, purified BFA-PG was obtained as described (Fig. 4) and chromatographed on Superose 6 after release of the HS-chains by alkaline elimination (A), after treatment with 0.01 mM CuCl$_2$ and 1 mM L-ascorbate followed by release of the HS-chains (B), after addition of 1 mM CuCl$_2$, dialysis against PBS, treatment with 0.5 mM SNP and 1 mM L-ascorbate followed by release of HS chains (C), after addition of both 1 mM CuCl$_2$ and 0.5 mM SNP, dialysis against PBS, treatment with 1 mM L-ascorbate followed by release of HS chains (D) and after addition of both 1 mM CuCl$_2$ and 0.5 mM SNP, recovery by using anti-SNO antiserum, treatment with 1 mM L-ascorbate followed by release of HS chains (E). $^3$H (o-----o).

FIG. 6. Release of NO from SNO groups by ascorbate and reformation of SNO in the presence of NO-donor and Cu$^{2+}$ (A), ascorbate-generated cleavage at GlcNH$_3^+$ with the concomitant appearance of anMan-terminating HS-oligosaccharides (B), colocalization of SNO- and GlcNH$_3^+$–containing epitopes (C) and absence of anMan-terminating HS-oligosaccharides in untreated cells (D). Confocal laser immunofluorescence staining of subconfluent cells for S-nitrosylated cysteines (SNO-Cys), a GlcNH$_3^+$-specific HS-epitope (JM-403), anMan-containing HS-fragments (AM) and Gpc-1 (GPC) in the absence or presence of ascorbate and after subsequent treatment with NO-donor and Cu$^{2+}$ showing in (A) that ascorbate destroys SNO-containing epitopes (SNO-Cys + Ascorbate) which can subsequently be restored (SNO-Cys + Ascorbate + SNP + CuCl$_2$), in (B) that treatment with ascorbate results in disappearance of the GlcNH$_3^+$-specific HS-epitope (JM-403 + Ascorbate) with concomitant appearance of the anMan-specific epitope (AM + Ascorbate), in (C) that S-nitrosylated Gpc-1 colocalizes with the GlcNH$_3^+$-specific HS-epitope in untreated cells.
(Merged) and in (D) that the anMan-specific epitope is undetectable in untreated cells (AM, counterstained for Gpc-1, GPC). In the two right panels of (B) the cells are visualized against a blue background. Bar, 20 µm.

SCHEME 1. HS-degradation during recycling of Gpc-1 in cells with upregulated polyamine-uptake. Position 1, Gpc-1 with three HS side-chains carrying GlcNH$_3^+$ residues mainly clustered near the core protein and sulfated uronic acids (HexUA-SO$_4$) mainly in peripheral regions. Position 2, Gpc-1 from cells treated with DFMO carrying HS chains with an increased number of GlcNH$_3^+$ residues and sulfated uronic acids, often juxtaposed. Position 3, Gpc-1 after degradation by heparanase and NO giving rise to core protein with truncated HS side-chains and free HS oligosaccharides, some terminating with anMan. Position 4, recycling Gpc-1 used for resynthesis of HS chains.

SCHEME 2. Model of Gpc-1 with intrinsic SNO groups generating NO for the deaminative cleavage of its own HS chains. Gpc-1 core protein contains cysteines that are substituted with NO derived from arginine. A redox reaction involving Cu$^{2+}$ to Cu$^{+}$ reduction is required for S-NO formation, which is presumably a relatively slow process. NO release also requires a redox reaction, here supplied by conversion of ascorbate to dehydroascorbate. The NO species formed can be NO$^+$, NO$^-$ or NO$^-$ depending on the redox state. The nitroxyl anion should be particularly reactive with the GlcNH$_3^+$ residues, generating anMan-terminating HS-oligosaccharides. Formation of the nitroxyl anion by reduction of the NO radical could be coupled to a Cu$^{+}$ to Cu$^{2+}$ oxidation which would complete a Cu-ion redox cycle. Regeneration of Cu$^{2+}$ can also be achieved by a number of other intracellular redox systems. NO-release and subsequent deaminative cleavage of HS should be a rapid reaction as both NO and HS are substituents on the same protein molecule.
Figure 1

Radioactivity ($10^{-2}$ x dpm)

Tube number
Figure 2
Figure 3

Tube number

Radioactivity ($10^2 \times$ dpm)

A

B

Figure 3
Figure 4
Figure 5
Figure 6
HS

\[ \text{Core} \]

1

\[ S \]

\[ \star = \text{GlcNH}_3^+ \]

2

\[ S = \text{HexUA-SO}_4 \]

\[ S \]

\[ \star = \text{Heparanase} \]

3

\[ S \]

\[ \star = \text{NO/NO}_2^- \]

4

\[ \text{Resynthesis} \]

Scheme 1
Scheme 2
Copper-dependent autocleavage of glypican-Iheparan sulfate by nitric oxide derived from intrinsic nitrosothiols

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